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Soil Microbes Determine Outcomes of Pathogenic Interactions Between *Radopholus similis* and *Fusarium oxysporum* V5w2 in Tissue Culture Banana Rhizospheres Starved of Nitrogen, Phosphorus, and Potassium

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The contributions of soil biota toward outcomes of pathogenic interactions between *Radopholus similis* and *Fusarium oxysporum* V5w2 in tissue culture banana plants starved of nitrogen (N), phosphorus (P), and potassium (K) were investigated. The study was based on three screenhouse factorial experiments ($2 \times 2 \times 2$) comprising of potted banana plants with or without *R. similis*, with or without *F. oxysporum* V5w2, and either grown in sterile or non-sterile soil. All plants in each of the three experiments received nutrient solutions that were deficient in N, P, or K, respectively. In all the three nutritional regimes, plants inoculated with *R. similis* were heavily colonized by the nematode with high percentage dead roots and necrosis, while their root biomasses were low. N-starved plants co-inoculated with *R. similis* and *F. oxysporum* V5w2 had lower percentage dead roots and tended to have numerically lower nematode density compared to those treated with *R. similis* only, especially in non-sterile soil. N-starved plants inoculated with *R. similis* had higher shoot dry weight, were taller with more leaves that were larger, compared to those not inoculated with the nematode. Plants grown in non-sterile soil had lower percentage dead roots, necrosis and *R. similis* density than those from sterile soil, regardless of the nutrient regime. N-starved plants from non-sterile soil were shorter with smaller leaves having decreased chlorophyll content and lower biomass, compared to those from sterile soil. By contrast, P and K starved plants from non-sterile soil were taller with larger leaves and more biomass, compared to those from sterile soil. Roots inoculated with *R. similis* had higher endophytic colonization by *Fusarium* spp., especially when co-inoculated with *F. oxysporum* V5w2 and grown in sterile soil among the N and K-starved plants. In conclusion, pathogenic interactions between *R. similis* and *F. oxysporum* V5w2 are predominantly suppressed by a complex of soil microbes that exert plant growth promoting effects in tissue culture banana plants through N, P, and K dependent processes. Nitrogen is the most important limiting

factor in rhizosphere interactions between banana roots, beneficial microbes and the pathogens. Soil sterilization and the stringent aseptic tissue culture techniques still require the development of alternative innovative ways of conserving microbial services for sustainable agriculture.

Keywords: *Fusarium oxysporum* V5w2, nitrogen-phosphorus-potassium, *Radopholus similis*, rhizosphere microbes, soil sterilization, tissue culture

INTRODUCTION

Soil-borne microbial and invertebrate pests and diseases are a major problem to crop production around the world. Of particular importance are disease complexes involving plant-parasitic nematodes and numerous fungal pathogen strains that cause root rots and wilt diseases (Back et al., 2002; Zhang et al., 2020). In banana production, *Radopholus similis* is among a complex of plant-parasitic nematodes that interact with the Fusarium of Banana Complex (FOBC) in causing root rots and wilt diseases (Speijer and Sikora, 1993; Ochieno, 2020; Rocha et al., 2020). Control of such disease complexes has focused on developing pest and disease resistant banana varieties (Tripathi et al., 2017; Siamak and Zheng, 2018), and multiplying them as clean planting material through aseptic cell and tissue culture technologies (Almekinders et al., 2019; Jacobsen et al., 2019).

Aseptic tissue culture protocols target the elimination of complexes of non-beneficial endophytic and soil-derived biota that are associated with plant growth suppression (Cassells, 2012; Kumar et al., 2021). Endophytic and rhizosphere plant growth promoting microbes are re-introduced to restore their beneficial functions lost through aseptic tissue culture and soil sterilization techniques (Niere et al., 1999; Sikora and Pocasangre, 2006; Sikora et al., 2008; Soumare et al., 2021). For instance, *Fusarium oxysporum* strain V5w2 is among endophytic microbes that have successfully been re-introduced into tissue culture banana plants and transferred to farmers in East Africa (Dubois et al., 2006, 2011; zum Felde, 2011). Induced systemic resistance is among the key mechanisms said to be involved in pest suppression by *F. oxysporum* V5w2 among other microbial biological control agents (Vu et al., 2006; Paparu et al., 2007, 2009a,b, 2010; Bubici et al., 2019; Damodaran et al., 2020). However, future research is still necessary to elucidate the mechanisms underpinning nematode suppression and increased banana yield observed after treatment with *F. oxysporum* V5w2 (Kisaakye et al., 2022). This is partly because *F. oxysporum* V5w2 has been reported to be a non-beneficial endophyte that interacts with *R. similis* in a wilt disease complex of banana (Ochieno, 2020, 2021).

Assessments of the improved banana varieties for resistance to soil-borne pests and diseases, as well as the function of plant growth promoting microbes, have been primarily performed through controlled screenhouse experiments utilizing potted plants and sterilized soil substrates (Dubois et al., 2004; Paparu et al., 2009b; Waweru et al., 2013). This way, integrated pest management (IPM) strategies for banana are developed.

Experiments with potted plants have often produced varied results. For instance, infections by strains of *Fusarium oxysporum*

have been associated with effects ranging from being positive, neutral, or negative toward the host banana plants and nematode pests (Athman et al., 2007; Paparu et al., 2009b; Ochieno, 2010, 2020). Studies that only feature potted banana plants and nematode pests in sterile soil fall short of information on the contribution of fungal pathogens toward parasitism (Hasan, 1993; Ochieno, 2010, 2020). This is also the case for studies that assess potted banana plants and fungal pathogens in sterile soil without plant-parasitic nematodes (Ochieno, 2010, 2020; Khan and Sharma, 2020). These two previous scenarios fail to address the natural interactions between plant-parasitic nematodes and fungal pathogens in root disease complexes. Moreover, possible roles of rhizosphere biota that exist in agroecosystems cannot be addressed through experiments that only feature sterilized soil (Ochieno, 2010, 2020; Kuijken et al., 2015; Jayaraman et al., 2021). Sterile soil may still be recolonized by different microbes (Li et al., 2019), while the axenic explants may still contain endophytic microbes or get re-infected (Ochieno, 2010, 2020; Thomas and Sekhar, 2017; Kithaku et al., 2019).

Abiotic factors especially nutrient compounds have been included into experiments to clarify their effects on interactions between plants, antagonistic and beneficial soil biota. This includes approaches that involve nutrient omission experimental treatments (Kolawole et al., 2018; Mugo et al., 2021). Where nutrient uptake by potted plants is not addressed, depletion of such resources could lead to variations in results. Comparison of plant-microbe-nematode interactions under different nutrient regimes produces varied results with different interpretations (Ochieno, 2010). Although differences in nutrient content and concentration are usually assumed, variations in other physico-chemical properties of the formulations including pH, salinity, conductivity, and toxicity may also affect the outcomes. Therefore, there is dilemma on how results from experiments utilizing nutrient formulations varying in multiple physico-chemical attributes should be interpreted (Ochieno, 2010). This is even further confounded by changes in soil physico-chemical composition that result from sterilization (De Deyn et al., 2004; Gundale et al., 2019; Dietrich et al., 2020). Experiments on plant-microbe-nematode interactions would therefore be better interpreted when conducted under a standard nutrient regime (Lancien et al., 1999; Shin et al., 2005; Ochieno, 2010), while addressing the contribution of rhizosphere microbes (De Rooij-van der Goes, 1995; Troelstra et al., 2001; Ochieno, 2010, 2020).

The present work is part of studies that are aimed at providing information on interactions between plant-parasitic nematodes and fungal pathogens, with specific focus on *R. similis* and *F. oxysporum* V5w2 in tissue culture banana (Ochieno, 2020).

The studies explore how the two root parasites interact with soil biota under nitrogen (N), phosphorus (P), and potassium (K) deficient conditions. It is hypothesized that, pathogenic interactions between *R. similis* and *F. oxysporum* V5w2 under N, P, and K deficient conditions are enhanced by a complex of non-beneficial soil microbes that exert plant growth suppressive effects in tissue culture banana plants.

MATERIALS AND METHODS

Experimental Design

Three experiments were conducted that comprised full factorial treatments ($2 \times 2 \times 2$) derived from banana plants either inoculated with *F. oxysporum* V5w2 or not, inoculated with *R. similis* or not, and either grown in sterile or non-sterile soil, hence resulting in eight treatment combinations (Ochieno, 2010). Each of the eight treatment combinations had 15 plants repeated twice over time as the replicates. The banana plants were completely randomized in the screen house. All plants in the three experiments were either supplied with nitrogen-deficient, phosphorus-deficient, or potassium-deficient nutrient solution, respectively.

Banana Plants

Tissue culture banana plants (genomic group AAA-EA, cv. *Kibuzi*; Onguso et al., 2004; Akankwasa et al., 2021), were obtained from the Tissue Culture Laboratory at the Sendusu Field Station of the International Institute of Tropical Agriculture in Uganda (IITA-Uganda). The plants had been micro-propagated by the shoot-tip culture technique in which plants are produced by multiplication of the meristematic tissue of banana corms (Vuylsteke, 1998). The banana plants had been maintained for 6 weeks in nutrient solution (1 g/L, Polyfeed™, Haifa Chemicals, Israel), after which they were graded into four sizes and equally distributed to the eight treatment groups of 15 plants. Four groups were marked for inoculation with *F. oxysporum* V5w2 and the other four were controls.

Pre-planting initial plant growth parameters were recorded through a slight modification of the method described by Akello et al. (2007) and Paparu et al. (2009b). Initial plant height (the distance from the base of the plant to the youngest leaf axil), initial number of open leaves, and initial width (widest part of the lamina) and initial length (the distance from the leaf apex to the leaf stalk) of the largest open leaf were recorded using a measuring tape. Initial fresh weight of each plant was measured using a toploading laboratory balance (Mettler Toledo™, Columbus, Ohio, United States), and the number of healthy-looking functional roots recorded.

Pre-planting initial plant growth-related parameters (mean \pm SE) in the three experiments did not vary between the treatments. In the experiment with N-starved banana plants, the initial measurements were fresh weight (8.5 ± 0.24 g), plant height (5.3 ± 0.15 cm), number of leaves (3.3 ± 0.05), leaf length (12.4 ± 0.19 cm), leaf width (4.8 ± 0.08 cm), and number of functional roots (3.6 ± 0.07) ($n = 340$, *F*-test, $p > 0.05$). In the experiment with P-starved banana plants, the initial measurements were fresh weight (6.9 ± 0.20 g), plant height (5.5 ± 0.10 cm), number

of leaves (3.7 ± 0.04), leaf length (11.2 ± 0.20 cm), leaf width (4.1 ± 0.10 cm), and number of functional roots (3.6 ± 0.08) ($n = 350$, *F*-test, $p > 0.05$). In the experiment with K-starved banana plants, the initial measurements were fresh weight (8.1 ± 0.20 g), plant height (5.6 ± 0.12 cm), number of leaves (3.3 ± 0.03), leaf length (12.7 ± 0.17 cm), leaf width (4.1 ± 0.10 cm), and number of functional roots (4.9 ± 0.08) ($n = 376$, *F*-test, $p > 0.05$).

Fungal Inoculum, Nematodes, and Soil

The study utilized *F. oxysporum* V5w2 (*nit 3*), a fungal strain proposed as a biological control agent that suppresses banana pests through induced systemic resistance (Dubois et al., 2011; Kabuye et al., 2011; zum Felde, 2011; Paparu et al., 2013), but has also been found to express mixed pathogenicity in banana plants (Ochieno, 2010, 2020). This fungal strain was originally obtained from a symptomless banana plant in Uganda by Schuster et al. (1995). The fungal inoculum had been maintained on sterile soil culture in the Laboratory of Microbiology at the Sendusu Field Station (IITA-Uganda). Inoculum was prepared by sprinkling the soil culture onto 90 mm diameter Petri dishes containing half-strength PDA (19 g/L, Sigma-Aldrich, Germany). Ten days later, the fungal spores and mycelia were scraped from the media into a 500 mL beaker containing sterile water. The spore mixture was sieved and homogenized, and then adjusted to 1.5×10^6 spores/mL.

Radopholus similis were originally obtained from banana fields at Sendusu Field Station (IITA-Uganda) and the culture was maintained aseptically on carrot discs (Speijer and De Waele, 1997). Nematodes for the experiment were supplied in sterile water suspension containing mixed stages of ~ 250 *R. similis*/mL.

Loamy soil was collected within a depth of 10 cm from a 5-year-old unmulched banana field at Sendusu Field Station (IITA-Uganda). The soil was sieved (5 mm aperture) before being thoroughly mixed. Soil samples were analyzed for chemical properties at the National Agricultural Research Laboratories (NARL) in Kawanda, Uganda. Available P and exchangeable K were extracted using the Mehlich-3 method (Mehlich, 1984). Phosphorus (P) in the extract was determined using the molybdenum blue colorimetric method and K using a flame photometer (Okalebo et al., 2002). Total N was analyzed by Kjeldahl oxidation and semi-micro Kjeldahl distillation (Bremner, 1960). Organic matter (OM) was determined using the Walkley-Black method (Walkley, 1947). Soil pH was analyzed using deionized water with a soil to water ratio of 1:2.5. The soil contained N (0.15 %), P (3.6 ppm), K (149 ppm i.e., 0.38 cmol_c/kg), OM (2.4 %), and pH 5.1. Part of the loamy soil was steam-sterilized at 100°C for 1 h using an electrode steam conditioner (Marshall-Fowler, South Africa). Analysis of soil chemical properties was not done after sterilization.

Inoculation and Planting

Banana plants were inoculated with *F. oxysporum* V5w2 by the root-dipping technique (Paparu et al., 2006, 2009a), but without prior breaking of the root tips (Ochieno, 2010). The roots were immersed into a 1.5 L spore suspension contained in tubs (30 × 25 × 15 cm, LWH) for 4 h. Control plants were immersed in distilled water. The plants were grown in 2.5 L buckets with

TABLE 1 | Chemical contents (milligrams) of complete nutrient solution (CNS) and those deficient in nitrogen (N), phosphorus (P) and potassium (K).

Chemical compounds	CNS	N-deficient	P-deficient	K-deficient
NH ₄ NO ₃	1,650	1,650	1,650	2,402
KNO ₃	1,900	1,900	1,900	.
KCl	.	401	93	.
CaCl ₂ .2H ₂ O	440	440	440	440
MgSO ₄ .7H ₂ O	370	370	370	370
KH ₂ PO ₄	170	170	.	.
NaH ₂ PO ₄ .H ₂ O	.	.	.	171
KI	0.83	0.83	0.83	0.83*
H ₃ BO ₃	6.2	6.2	6.2	6.2
MnSO ₄ .4H ₂ O	22	22	22	22
ZnSO ₄ .7H ₂ O	8.6	8.6	8.6	8.6
Na ₂ MoO ₄ .2H ₂ O	0.25	0.25	0.25	0.25
CuSO ₄ .5H ₂ O	0.025	0.025	0.025	0.025
CoCl ₂ .6H ₂ O	0.025	0.025	0.025	0.025
Na ₂ EDTA.2H ₂ O	37.3	37.3	37.3	37.3
FeSO ₄ .7H ₂ O	27.8	27.8	27.8	27.8

Complete nutrient solution (CNS) based on Murashige and Skoog (1962); the other three solutions were made by omission of N, P, or K from the CNS respectively.

*In the K-deficient treatment this negligible amount of potassium (K) was retained due to unavailability of alternative source of iodine (I).

6 holes at the base containing either sterile or non-sterile soil under screen house conditions ($25 \pm 3^\circ\text{C}$, 70–75% RH, 12L: 12D photoperiod). Initial plant heights were recorded. Each plant was supplied with rain water (100 mL) daily, and the respective N-deficient, P-deficient, and K-deficient nutrient solutions (100 mL) once every week. The N, P, and K-deficient nutrient solutions were formulated by omitting the respective elements from the chemical ingredients in the solution prepared by Murashige and Skoog (1962) (Table 1). Twenty (20) days after planting, three holes (5 cm deep) were made into the soil around the base of each plant using a disinfected stick. Each plant was inoculated with ~ 500 female *R. similis* in 2 mL of the nematode suspension, distributed across the three holes before covering them with soil.

Plant Growth-Related Parameters

Sixty days after inoculation with *R. similis* chlorophyll content in the youngest open leaf of every banana plant was measured in SPAD units using a Chlorophyll Meter (SPAD 502, Spectrum Technologies, Illinois, USA; Reeves et al., 1993; Bakaze et al., 2021). Chlorophyll content was only measured in N-starved plants. At 100 days after nematode inoculation, plant height, number of leaves, length and width of youngest open leaf were recorded. Plants were harvested and the number of dead and healthy roots were recorded and used to calculate the percentage dead roots. The shoots and roots were detached and their fresh weights recorded. Root and shoot biomasses were combined for total plant biomass. The shoots were oven-dried at 70°C for a discontinuous period of 14 days to determine their dry weight. The roots were preserved at 4°C awaiting the extraction of nematodes and root-invading microbes. Five roots per plant

were randomly selected for assessment of necrosis caused by nematodes, and for estimation of nematode densities. Three roots were randomly selected per plant to estimate the level of endophytic colonization by *F. oxysporum* V5w2.

Root Necrosis Assessment and Nematode Densities

Root necrosis assessment and quantification of nematode densities were undertaken based on the methods of Speijer and De Waele (1997) and Brooks (2004). Each of the five roots selected per plant for necrosis assessment was cut into 10 cm length. The pieces were then split longitudinally and one half selected from each of them. The cumulative length of necrotic lesions of cortical tissue on each 10 cm side of the stele was recorded as a fraction of the total 20 cm length per root. The sum length of the necrotic tissue for five roots per plant was then expressed as percentage necrosis. These roots were chopped into small pieces and 5 g sub-samples were mixed with 50 mL of water, and macerated at medium speed for 20 s using a Waring® laboratory blender. Nematodes were extracted from the suspension using a Baermann tray for 24 h. The extracts were transferred into 100 mL glass bottles and stored at 4°C for 24 h to allow the nematodes to sediment. The water volume was standardized to 25 mL by gentle siphoning from the surface. The suspension was homogenized by stirring, and nematodes characterized based on identification keys (Fortuner, 1988; Luc et al., 2005), while being quantified on a counting dish at $\times 10$ magnifications from triplicate 2.5 mL pipette sub-samples. Densities of *R. similis* were expressed as counts per 100 g of root material, while other identified nematode species were not quantified.

Endophytic Colonization by *F. oxysporum* V5w2

Each of the three roots selected for estimation of endophytic colonization by *F. oxysporum* V5w2 was surface-sterilized by dipping it into 96% ethanol followed by flaming. This ensured that only microbes within the tissues remained alive. Each root was then cut into six pieces. The root pieces were plated on 60 mm glass Petri dishes containing PDA enriched with KClO₃ for 7 days. Growth of *Fusarium* strains including V5w2 on PDA is characterized by whitish mycelia with pink pigmentation (Leslie and Summerell, 2008). The number of root pieces with growth of *Fusarium* species among six assessed pieces was recorded. Root piece colonization was the percentage of all root pieces yielding *Fusarium* among all assessed pieces within a treatment. Other root-invading microbes growing from the root pieces were subcultured and morphologically identified through the TSBF-CIAT project on Below-Ground Biodiversity (BGBD) funded by GEF-UNEP at the Soil Microbiology Laboratory, Makerere University, Uganda (Nakintu et al., 2008; Ochieno, 2010).

In vitro growth inhibition tests between the microbes and *F. oxysporum* V5w2 were conducted. *Fusarium oxysporum* V5w2 was inoculated in the center of a glass Petri dish containing PDA using a sterile needle. The microbes were inoculated on four spots surrounding the fungal pathogen. The fungal pathogen or the test

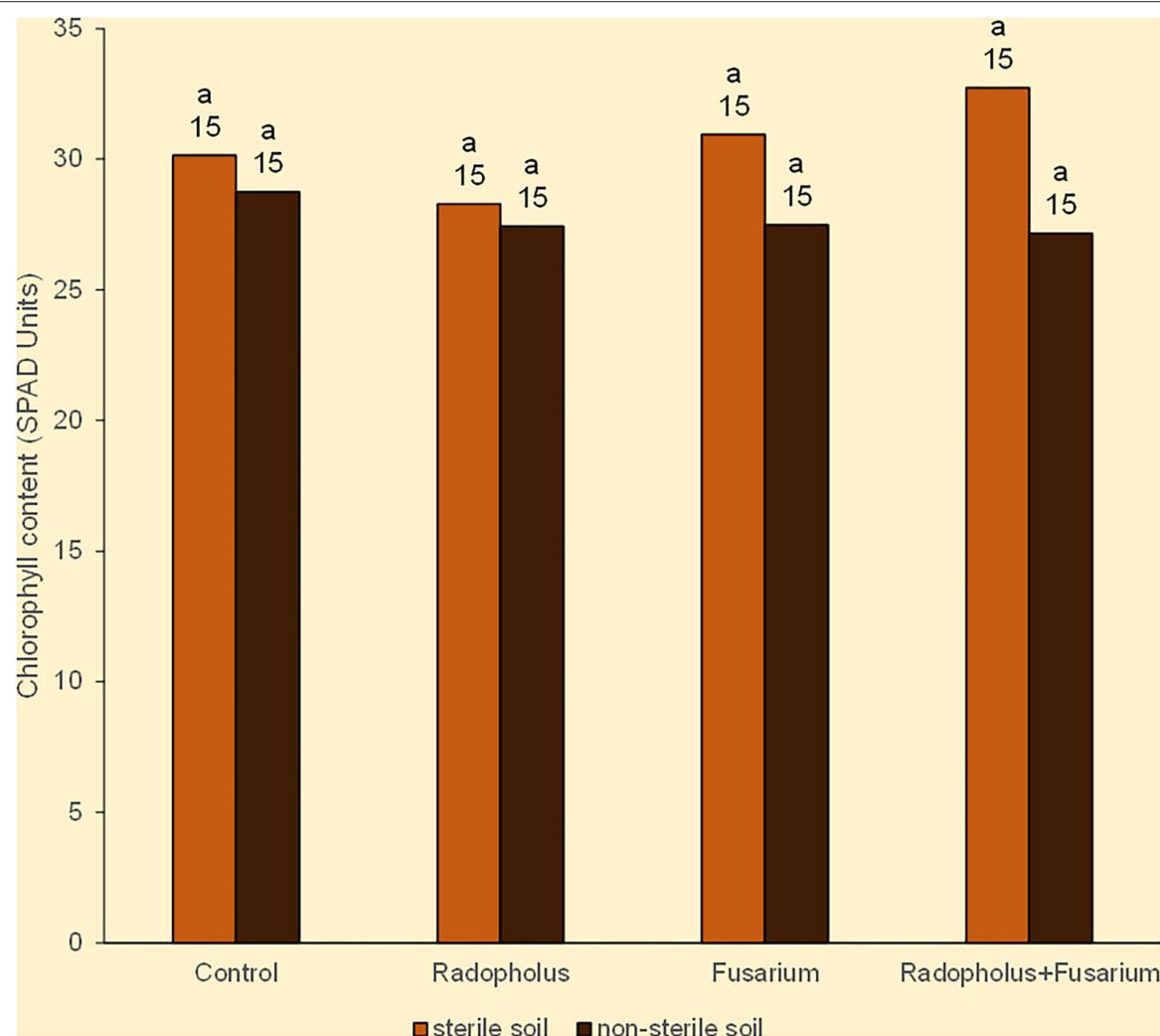


FIGURE 1 | Chlorophyll content in Nitrogen-starved tissue culture banana plants subjected to independent or combined inoculation with *Radopholus similis* and the *Fusarium oxysporum* V5w2 and grown in sterile or non-sterile soil. Bars with the same letter represent values that are not statistically different, with the sample sizes indicated below the letters (χ^2 -test, $p < 0.05$).

microbe was considered inhibitive if its mycelia grew into the colonies of the other marked with a boundary between them.

Optical Density of Root Extracts

In the experiment with N-starved plants, it was realized that roots that were stored at 4°C exhibited differences in color after 3 days. Therefore, visual and colorimetric tests on roots and their extracts were conducted to assess possible effects of the experimental treatments on root color. Roots from all plants per treatment were mixed. This was followed by random selection of up to 30 roots from every treatment; the color of the external root surface was visually recorded as either brown or light-brown by comparing with two roots that had been pre-selected as standards for the two colors. For colorimetric assessments, five roots from three plants per treatment were chopped together into a composite sample. This was conducted in triplicate. Ten grams (10 g) of each composite sample were mixed with 100 mL distilled

water, and macerated using a Waring® Laboratory Blender (Christison Scientific, Gateshead, UK). The root extracts were diluted (10×) and their optical density (absorbance) measured at 320 nm using a spectrophotometer (Genesys 10 UV, Thermo Fisher Scientific, USA).

Statistical Analysis

Data were analyzed using SAS 9.1 software (SAS, 2004). Means were generated by Proc Means. Diagnostic check for normality was conducted using Proc Univariate. Proc Transreg was used to find appropriate Box-Cox transformations, which included generation of suitable powers for data that required transformation, with particular reference to root biomass, total biomass, shoot fresh and dry weight, plant height, number and size leaves. Percentage dead roots and percentage necrosis (x) were arcsine $\sqrt{(x/100)}$ transformed, while untransformed data were used for nematode densities, chlorophyll content and

TABLE 2 | Effect of soil sterility on infection symptoms of *Radopholus similis* and *Fusarium oxysporum* V5w2 in tissue culture banana plants grown under nitrogen deficient conditions.

Source of variation	Df	Root damage		<i>R. similis</i>	Biomass: freshweight (fwt) and dryweight (dwt)				Height	Leaves				
		Death	Necrosis		Root fwt	Shoot fwt	Shoot dwt	Total fwt		Number	Length	Width		
<i>F</i> -values														
Main effects														
Nematode <i>R. similis</i> (Rs)	1	978.4***	1,237.3***	151.2***	29.4***	3.1	6.5*	1.4	9.9*	4.8*	9.6**	9.9**		
Fungus <i>F. oxysporum</i> (Fo)	1	5.8*	0.1	5.9*	1.0	0.7	0	0	0	1.3	0.6	0.6		
Soil sterility (S)	1	31.4***	4.5*	35.4***	33.3***	166.0***	71.1***	125.6***	59.6***	0	65.9***	65.2***		
Two-way interactions														
Rs × Fo	1	4.0*	0.6	3.5	1.9	0	1.3	0.3	3.1	4.1*	3.0	3.0		
Rs × S	1	69.9***	69.8***	32.4***	22.2***	1.8	0.4	9.1**	0.9	9.5**	1.4	1.1		
Fo × S	1	5.1*	0.6	0.3	0.2	0	1.7	0	0.1	0.6	1.2	1.2		
Three-way interactions														
Rs × Fo × S	1	18.0***	3.1	0.1	4.1*	1.0	0.1	2.2	0.1	0.2	0.2	0.2		
c.v.		50.2	43.4	139.7	40.2	32.5	20.7	34.0	30.1	11.0	18.3	21.7		
Total (df)	7	326	326	322	326	326	326	326	328	328	326	326		
					Means									
		percent	percent	count	gram	gram	gram	gram	cm	number	cm	cm		
Grand mean		15.0	23.9	12,546	52.8	130.9	13.2	183.7	24.9	3.8	31.1	14.2		
Nematode	<i>R. similis</i>	28.8 a	45.9 a	24,187 a	46.4 b	133.9	13.9 a	180.4 a	25.7 a	3.9 a	31.8 a	14.7 a		
	Non	1.1 b	1.9 b	619 b	59.2 a	127.9	12.5 b	187.1 a	24.0 b	3.7 b	30.3 b	13.6 b		
Fungus	<i>F. oxysporum</i>	14.3 b	24.2	10,372 b	54.0	129.6	13.2	183.5	24.9	3.7	30.9	14.1		
	Non	15.7 a	23.7	14,773 a	51.6	132.3	13.2	183.9	24.8	3.8	31.2	14.3		
Soil	Sterile	19.0 a	27.7 a	18,237 a	59.5 a	155.2 a	15.6 a	214.6 a	27.0 a	3.8	33.1 a	15.0 a		
	Non-sterile	10.9 b	20.0 b	6,855 b	46.0 b	106.1 b	10.8 b	152.1 b	22.6 b	3.8	29.0 b	13.3 b		
<i>R. similis</i>	<i>F. oxysporum</i>	27.4 b	45.9	20,320	49.1	132.3	13.5	181.4	25.3	3.9 a	31.2	14.5		
	Non	30.3 a	46.0	28,195	43.6	135.7	14.3	179.2	26.2	3.8 ab	32.4	15.0		
No <i>R. similis</i>	<i>F. oxysporum</i>	1.0 c	2.1	60	58.9	126.8	12.9	185.7	24.5	3.5 b	30.6	13.7		
	Non	1.3 c	1.7	1,185	59.5	129.0	12.1	188.5	23.4	3.8 ab	30.1	13.5		
<i>R. similis</i>	Sterile	37.5 a	55.5 a	35,393 a	47.6 b	155.4	16.3	203.0 b	27.6	3.7 ab	33.5	15.3		
	Non-sterile	20.0 b	36.0 b	13,114 b	45.2 b	112.2	11.5	157.4 c	23.9	4.0 a	30.1	14.2		
No <i>R. similis</i>	Sterile	0.6 c	0.1 d	872 c	71.2 a	154.9	15.0	226.1 a	26.5	3.8 ab	32.7	14.7		
	Non-sterile	1.7 c	3.8 c	363 c	46.8 b	99.9	10.0	146.7 c	21.4	3.5 b	27.9	12.5		
<i>F. oxysporum</i>	Sterile	19.6 a	29.0	15,733	60.0	154.0	16.0	214.0	27.2	3.7	33.2	15.0		
	Non-sterile	8.7 b	19.0	4,947	47.8	104.2	10.4	151.9	22.5	3.8	28.5	13.1		
No <i>F. oxysporum</i>	Sterile	18.2 a	26.3	20,834	58.9	156.3	15.2	215.2	26.9	3.9	33.0	15.0		
	Non-sterile	13.1 a	21.0	8,786	44.2	108.0	11.1	152.2	22.7	3.8	29.5	13.6		
<i>R. similis</i>	Sterile	Fo	39.3 a	57.8	30,705	47.5 b	152.2	16.2	199.7	27.1	3.8	33.1	15.2	

(Continued)

TABLE 2 | Continued

		Means								
		percent	percent	count	gram	gram	gram	cm	number	cm
No <i>R. similis</i>	Sterile	Non	35.6 a	53.2	40,432	47.8 b	158.8	16.3	206.6	28.0
	Non-sterile	Fo	15.1 c	33.5	9,688	50.9 b	111.9	10.9	162.7	23.4
	Non-sterile	Non	25.0 b	38.5	16,541	39.4 b	112.5	12.2	151.9	24.4
	Sterile	Fo	0 d	0.1	31	72.5 a	155.8	15.8	228.4	27.2
	Sterile	Non	1.3 d	0.1	1,713	69.8 a	153.9	14.2	223.7	25.8
	Non-sterile	Fo	2.1 d	4.3	90	44.6 b	96.3	9.9	140.9	21.7
	Non-sterile	Non	1.2 d	3.4	644	49.0 b	103.4	10.1	152.4	21.1
									3.6	28.0
										12.5

Asterisks indicate significant effect (** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$; Means for significant effects are indicated in bold font; those with the same letter are not significantly different ($p > 0.05$).

optical density of root extracts. Proc GLM was used for three-way factorial analyses of variance (ANOVA) among the treatments. Mean separation was done by *t*-tests (LSD) when ANOVA was significant. Root piece colonization by *Fusarium* was analyzed by Proc Genmod (binomial distribution); Bonferroni-adjusted *p*-values were obtained by Proc Multtest from raw *p*-values in Proc Genmod, and used for pairwise comparisons between treatments. Root coloration data were analyzed by Proc Genmod and pairwise comparisons using contrasts.

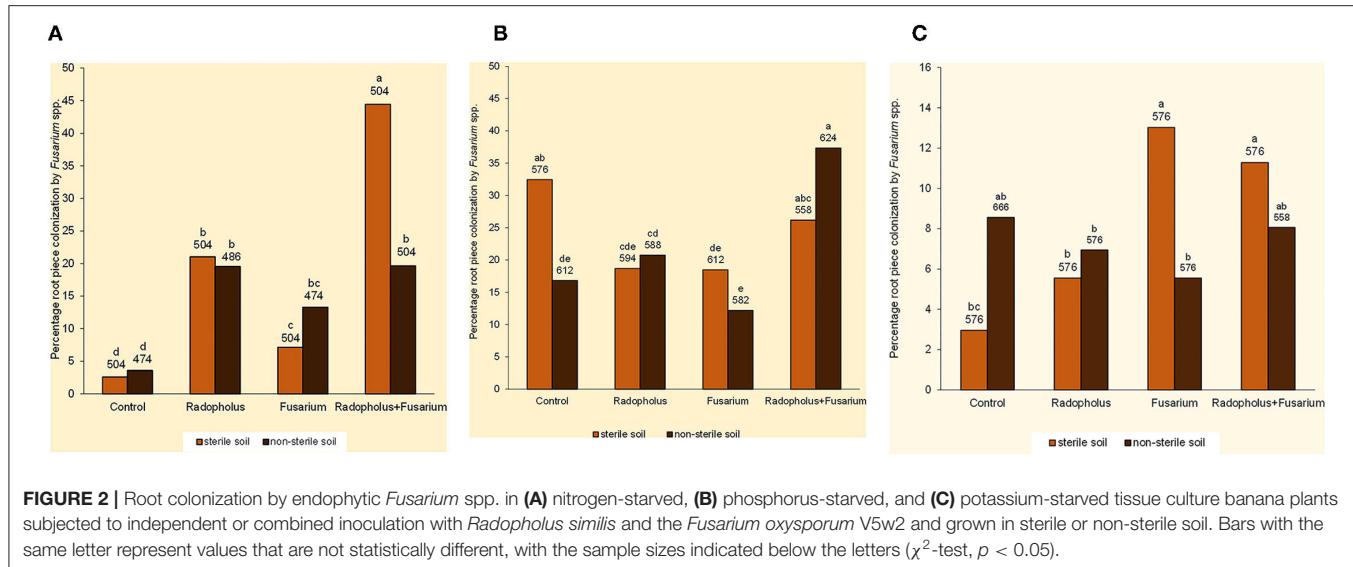
RESULTS

Experiment With N-Starved Tissue Culture Banana Plants

All plants expressed symptoms that may be linked to N-starvation in the form of being stunted with yellow leaves that had pinkish veins. The leaves dried from the margins toward the midribs, and wilted from the lower older ones, toward the upper young ones. The mean chlorophyll (nitrogen) content of all plants was 29 SPAD units. Plants that were inoculated with *R. similis* or *F. oxysporum* V5w2 visually appeared to have greener leaves than those without the nematode, with this tendency being weakly reflected in leaf chlorophyll contents (Figure 1). Plants grown in sterile soil had higher chlorophyll (nitrogen) content than those from non-sterile soil, scoring 31 and 28 SPAD units, respectively (Figure 1). Roots of the banana plants generally appeared light colored, were thin and considerably elongated to densely coil inside the pots, while the soil substrate became sticky and depleted.

Dead roots, necrosis and *R. similis* were mainly found in plants that had been inoculated with the nematode. Banana plants inoculated with *F. oxysporum* V5w2 had lower density of *R. similis* than those not inoculated with the fungal endophyte (Table 2). *R. similis*-inoculated plants grown in sterile soil had higher percentage dead roots than those from non-sterile soil (Table 2). Among *R. similis*-inoculated plants grown in non-sterile soil, those inoculated with *F. oxysporum* had a lower percentage dead roots than those not inoculated with the fungal endophyte. In contrast, among *R. similis*-inoculated plants grown in sterile soil, there was no difference in percentage dead roots between *F. oxysporum*-inoculated plants and those not inoculated with the fungal endophyte. *Radopholus similis*-inoculated plants grown in sterile soil had a higher percentage root necrosis and higher density of *R. similis* than those from non-sterile soil (Table 2). Thirteen other nematode species, identified mainly in non-sterile soil among the soil samples, included; the plant parasitic *Helicotylenchus multicintus*, *Meloidogyne* spp., *Pratylenchus goodeyi*, *Xiphinema* spp., *Trophorus* spp., *Scutellonema* spp., *Rotylenchus* spp., *Tylenchus* spp., *Hemicycliophora* spp., *Paratrichodorus* spp., and *Ditylenchus* spp. Saprophytic genera included *Aphelenchus* spp. and *Rhabditis* spp. among numerous others that could not be identified. The first three nematode species were frequently recorded in root samples from non-sterile soil.

Banana plants grown in sterile soil without *R. similis* inoculation had a higher root biomass compared to those



from the remaining treatments, which had similar root biomass (**Table 2**). Plants from sterile soil had higher total biomass than those from non-sterile soil. In sterile soil, *R. similis*-inoculated plants had lower total biomass than those without nematodes. However, in non-sterile soil, total plant biomass did not vary between *R. similis*-inoculated and those not inoculated with the nematode.

Shoot dry weight, plant height and size of leaves had higher values in *R. similis*-inoculated plants than in those not inoculated with the nematode (**Table 2**). *F. oxysporum*-inoculated plants that received *R. similis* inoculum had more leaves than those that were not inoculated with the nematode. In non-sterile soil, plants that were inoculated with *R. similis* had more leaves than those not inoculated with the nematode. Shoot fresh and dry weights, plant height, length, and width of leaves were greater in plants from sterile than those from non-sterile soil (**Table 2**).

Percentage root piece colonization by *Fusarium* spp. was higher in banana plants that received independent and combined inoculations with *F. oxysporum* V5w2 and *R. similis* compared to the non-inoculated controls (**Figure 2A**). Those inoculated with *R. similis* had higher root piece colonization by *Fusarium* spp., especially when co-inoculated with *F. oxysporum* V5w2 and grown in sterile soil. Twenty-five other endophytic fungi were isolated from the banana root pieces with 11 genera among them identified and included; *Fusarium* spp., *Rhizoctonia* spp., *Trichoderma* spp., *Sclerotium* spp., *Bipolaris* spp., *Curvularia* spp., *Mucor* spp., *Rhizopus* spp., *Papulaspora* spp., *Pleurophragmium* spp., and *Trichocladium* spp. *In vitro* growth inhibition tests revealed the presence of *Fusarium* spp. and other microbes that interacted with *F. oxysporum* V5w2 with both antagonistic and neutral effects (Ochieno, 2010).

Darkened roots were associated with plants that were inoculated with *R. similis* and those that were grown in non-sterile soil (**Figure 3**). Optical density of root extracts exhibited small ranges of indiscernible variations that averaged 0.51 absorbance.

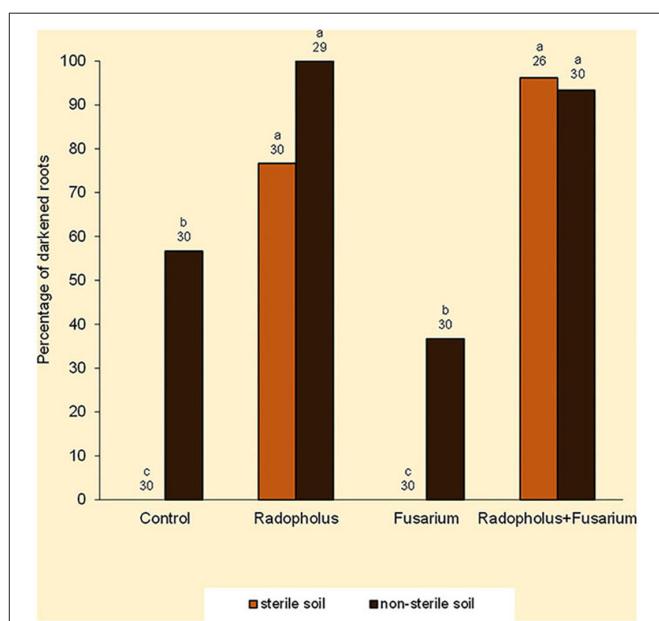


FIGURE 3 | Percentage of dark-colored roots in Nitrogen-starved tissue culture banana plants subjected to independent or combined inoculation with *Radopholus similis* and *Fusarium oxysporum* V5w2 and grown in sterile or non-sterile soil. Bars with the same letter represent values that are not statistically different, with the sample sizes indicated below the letters (χ^2 -test, $p < 0.05$).

Experiment With P-Starved Tissue Culture Banana Plants

Most plants exhibited visual symptoms that could be linked to P-starvation, which included dark-green leaves with slightly yellowing margins, while the older leaves curled downwards and dried. Dead roots were mainly found in *R. similis*-inoculated plants, and their percentage was higher in sterile than in non-sterile soil. Necrotic lesions were mainly found in plants that were

inoculated with *R. similis*, and the percentage root necrosis was higher in non-sterile than in sterile soil (**Table 3**).

Roots of plants co-inoculated with *R. similis* and *F. oxysporum* V5w2 had a higher density of the nematode than those that were not inoculated with the fungal pathogen (**Table 3**). Roots of nematode-inoculated plants from sterile soil had a higher density of *R. similis* than those from non-sterile soil (**Table 3**).

R. similis-inoculated plants in non-sterile soil had greater root biomass than those from sterile soil. *R. similis*-inoculated plants were shorter, had lower shoot fresh weight and total biomass than those without the nematode (**Table 3**). Plants in non-sterile soil were taller and had more total biomass than those from sterile soil. However, there was no difference in shoot dry weight between treatments (**Table 3**).

Plants that were grown in non-sterile soil produced more leaves than those from sterile soil (**Table 3**). Within sterile soil, *R. similis*-inoculated plants had fewer leaves than those without the nematode. However, in non-sterile soil, the number of leaves did not vary with *R. similis* treatment (**Table 3**). Leaves of plants that were grown in non-sterile soil were longer and wider than those from sterile soil. Plants inoculated with *F. oxysporum* V5w2 were generally smaller than those not inoculated with the fungus, although these differences were only of numerical significance (**Table 3**).

Fusarium spp. that were not visually different from the inoculated *F. oxysporum* V5w2 were observed in roots of all treatments and controls. Mycelia of *Fusarium* spp. that emerged from these roots appeared very sparse with scarcity of spores. In non-sterile soil, percentage root piece colonization by *Fusarium* spp. was highest in plants co-inoculated with *F. oxysporum* V5w2 and *R. similis* (**Figure 2B**). Trends in percentage root piece colonization by *Fusarium* spp. in sterile soil did not clearly reflect those in non-sterile soil (**Figure 2B**). Root pieces from the P-starved plants appeared more heavily contaminated with a variety of other microbes that were also isolated, especially from samples arising from non-sterile.

Experiment With K-Starved Tissue Culture Banana Plants

A symptom that could be linked to K-starvation, which was characterized by development of bright-orange coloration on leaf laminae, was evident in the K-starved plants. *R. similis*-inoculated plants in sterile soil had a higher percentage dead roots and higher density of the nematode than those from non-sterile soil (**Table 4**). Percentage root necrosis was higher in plants that were inoculated with *R. similis* than those without the nematode (**Table 4**).

Root biomass for *F. oxysporum* V5w2-inoculated plants was lower than for plants that were not inoculated with the fungal pathogen (**Table 4**). Root biomass for *R. similis*-inoculated plants that were grown in sterile soil was lower than for plants from non-sterile soil (**Table 4**). Shoot fresh weight, total biomass, plant height, leaf length, and width, were lower in plants that were inoculated with *R. similis* than in non-inoculated ones, and higher in non-sterile than in sterile soil (**Table 4**). Shoot dry weight was lower

in *R. similis*-inoculated plants than in the non-inoculated ones (**Table 4**).

Nematode-treated plants had fewer leaves than those without nematodes (**Table 4**). Plants that were inoculated with *F. oxysporum* V5w2 and grown in sterile soil had fewer leaves than those that were not inoculated (**Table 4**). Plants that were grown in non-sterile soil had more leaves than those from sterile soil (**Table 4**).

Percentage root piece colonization by *Fusarium* spp. was high in plants inoculated with *F. oxysporum* V5w2 and grown in sterile soil (**Figure 2C**). Percentage root piece colonization by *Fusarium* spp. was lower in non-sterile soil than in sterile soil among plants that received the fungal inoculum (**Figure 2C**).

DISCUSSION

The present three experiments were conducted to find out whether, pathogenic interactions between *R. similis* and *F. oxysporum* V5w2 under nitrogen (N), phosphorus (P), and potassium (K) limited conditions, are enhanced by a complex of non-beneficial soil microbes that exert plant growth suppressive effects in tissue culture banana (Kumar et al., 2021). Plant parasitic activities of *R. similis* on banana plants were evident in the form of increased density of the nematode that resulted in root damage through necrosis, death and reduced plant biomass. This root burrowing nematode is known to cause such root damage that inhibits water and nutrient uptake from the rhizosphere resulting in poor growth and toppling of banana trees (Devi et al., 2009; Haegeman et al., 2010; Seenivasan, 2017). Root damage by *R. similis* also allows co-infections by root-invading organisms such as microbial pathogens and other plant-parasitic nematodes involved in plant disease complexes (Khan and Sharma, 2020). Such root-invading organisms may have included the 25 endophytic fungi that were isolated from the banana root pieces among them *Fusarium* spp., *Rhizoctonia* spp., *Trichoderma* spp., *Sclerotium* spp., *Bipolaris* spp., *Curvularia* spp., *Mucor* spp., *Rhizopus* spp., *Papulaspora* spp., *Pleurophragmium* spp., and *Trichocladium* spp.; as well as plant-parasitic nematodes that included *Helicotylenchus multicintus*, *Meloidogyne* spp., *Pratylenchus goodeyi*, *Xiphinema* spp., *Trophorus* spp., *Scutellonema* spp., *Rotylenchus* spp., *Tylenchus* spp., *Hemicyclophora* spp., *Paratrichodorus* spp., and *Ditylenchus* spp.

Contrary to expectations, N-starved banana plants inoculated with *R. similis* exhibited better growth in terms of shoot biomass, height, number, and size of leaves. The N-starved banana plants inoculated with *R. similis* even tended to have higher chlorophyll content that made them appear less yellow or otherwise greener. Probably, N in the form of ammonium excreted by *R. similis* feeding on the roots is preferentially directed toward chlorophyll synthesis under N-deficient conditions. This is because the nematode-excreted ammonium (NH_4^+ ; Neher, 2001; Zinov'eva et al., 2004), is the most readily available form of nitrogen for assimilation by plants (Mur et al., 2017; Ochieno et al., 2021). Also, root-feeding activities of *R. similis* may have enhanced the rhizodeposition of carbon-rich compounds including enzymes

TABLE 3 | Effect of soil sterility on infection symptoms of *Radopholus similis* and *Fusarium oxysporum* V5w2 in tissue culture banana plants grown under phosphorus deficient conditions.

Source of variation	df	Root damage		<i>R. similis</i>	Biomass: freshweight (fwt) and dryweight (dwt)				Height		Leaves				
		Dead	Necrosis		Root fwt	Shoot fwt	Shoot dwt	Total fwt	Number	Length	Width				
<i>F</i> -values															
Main effects															
Nematode <i>R. similis</i> (Rs)	1	1,905***	1,599.2***	111.4***	81.7***	15.3***	2.7	28.4***	4.4*	33.1***	3.7	1.3			
Fungus <i>F. oxysporum</i> (Fo)	1	0	2.3	5.7*	0	2.1	2.6	0.9	1.8	2.6	1.5	0.6			
Soil sterility (S)	1	72***	9.9**	19.3***	37.2***	1.9	0.5	7.1**	17.6***	244.9***	11.7***	37.4***			
Two-way interactions															
Rs x Fo	1	0.8	0.7	6.5*	2.2	0.7	0.4	0.9	0	0.4	0	0.1			
Rs x S	1	90.3***	1.5	21.6***	8.9**	2.9	3.2	3.4	0.1	7.8**	0.8	0.3			
Fo x S	1	2.2	3.1	3.8	0	0.7	0.1	0.5	1.5	2.0	1.9	2.3			
Three-way interactions															
Rs x Fo x S	1	0.2	4.0	3.3	0.5	0.1	0	0.3	0.6	3.1	0	0.5			
c.v.		42.1	44.4	169.7	30.0	43.1	40.6	42.0	40.4	25.6	39.0	35.7			
Total (df)	7	343	341	337	343	344	344	343	344	344	344	344			
Means															
		percent	percent	Count	gram	gram	gram	gram	cm	count	cm	cm			
Grand mean		17.0	19.2	4,849	51.1	161.0	16.5	212.3	32.9	5.2	37.7	16.2			
Nematode	<i>R. similis</i>	33.9 a	38.5 a	9,510 a	41.8 b	148.9 b	16.1	191.1 b	31.8 b	5.0 b	36.8	16.0			
	non	0.2 b	0.3 b	161 b	60.3 a	173.1 a	17.0	233.4 a	33.9 a	5.5 a	38.5	16.4			
Fungus	<i>F. oxysporum</i>	17.3	18.3	5,896 a	51.5	156.5	16.1	208.4	32.3	5.3	37.3	16.1			
	non	16.8	20.1	3,820 b	50.7	165.4	17.0	216.2	33.4	5.2	38.0	16.3			
Soil	Sterile	22.3 a	17.6 b	6,725 a	44.8 b	156.2	16.7	201.5 b	30.8 b	4.6 b	36.3 b	15.3 b			
	Non-sterile	11.7 b	20.8 a	2,918 b	57.4 a	165.7	16.4	223.1 a	34.9 a	5.9 a	39.1 a	17.1 a			
<i>R. similis</i>	<i>F. oxysporum</i>	34.7	36.8	11,768 a	40.8	141.4	15.4	182.9	31.0	5.0	36.5	15.8			
	Non	33.2	40.1	7,330 b	42.9	156.4	16.7	199.2	32.5	4.9	37.2	16.2			
No <i>R. similis</i>	<i>F. oxysporum</i>	0	0.2	93 c	62.0	171.7	16.8	233.7	33.5	5.6	38.2	16.4			
	Non	0.4	0.4	230 c	58.6	174.5	17.3	233.1	34.3	5.4	38.8	16.4			
<i>R. similis</i>	Sterile	44.9 a	35.6	13,476 a	32.9 c	138.4	15.7	172.0	29.5	4.1 c	35.1	15.0			
	Non-sterile	23.1 b	41.3	5,498 b	50.7 b	159.3	16.4	210.0	34.1	5.8 a	38.6	17.0			
No <i>R. similis</i>	Sterile	0 c	0.1	51 c	56.6 ab	174.1	17.8	230.6	32.1	5.0 b	37.5	15.6			
	Non-sterile	0.4 c	0.5	277 c	64.0 a	172.2	16.3	236.2	35.7	6.0 a	39.5	17.2			
<i>F. oxysporum</i>	Sterile	23.2	15.3	8,610	45.2	154.0	16.3	200.0	30.7	4.6	36.3	15.4			
	Non-sterile	11.4	21.2	3,085	57.6	159.1	15.8	216.7	33.8	6.0	38.4	16.8			
No <i>F. oxysporum</i>	Sterile	21.5	19.9	4,862	44.4	158.5	17.1	202.9	30.8	4.6	36.3	15.2			
	Non-sterile	12.1	20.3	4,862	57.1	172.4	17.0	229.5	36.0	5.8	39.8	17.4			

Asterisks indicate significant effect (**p ≤ 0.001, *p ≤ 0.01, *p ≤ 0.05); Means for significant effects are indicated in bold font; those with the same letter are not significantly different (p > 0.05).

TABLE 4 | Effect of soil sterility on infection symptoms of *Radopholus similis* and *Fusarium oxysporum* V5w2 in tissue culture banana plants grown under potassium deficient conditions.

Source of variation	df	Root damage		<i>R. similis</i>	Biomass: freshweight (fwt) and dryweight (dwt)				Height		Leaves				
		Dead	Necrosis		Root fwt	Shoot fwt	Shoot dwt	Total fwt	Number	Length	Width				
<i>F</i> -values															
Main effects															
Nematode <i>R. similis</i> (Rs)	1	305.8***	252.6***	102.6***	77.5***	35.2***	12.3***	47.6***	12.7***	7.5**	17.1***	14.1***			
Fungus <i>F. oxysporum</i> (Fo)	1	0.4	0.3	2.2	11.2***	1.0	0	2.7	0.1	7.2**	0.8	0.4			
Soil sterility (S)	1	9.9**	0.1	11.1**	32.9***	12.1***	0.2	19.2***	29.6***	64.5***	52.4***	53.9***			
Two-way interactions															
<i>Rs</i> × <i>Fo</i>	1	0	0.1	1.9	2.1	0.7	0	1.3	0.2	0.4	0.5	0.8			
<i>Rs</i> × S	1	12.5***	0.6	16.7***	5.4*	2.1	0	2.3	2.0	2.3	1.5	1.4			
<i>Fo</i> × S	1	0.1	1.3	0.1	1.1	3.7	0.6	3.2	0.5	4.1*	0.7	0.6			
Three-way interactions															
<i>Rs</i> × <i>Fo</i> × S	1	0.5	3.5	0.3	0.3	0.5	0.5	0.2	0.1	3.8	0.5	0.7			
c.v.		106.6	105.7	173.6	17.2	31.8	23.5	25.9	19.5	26.3	28.8	18.5			
Total (df)	7	355	352	350	355	353	350	353	356	355	355	355			
Means															
		percent	percent	count	gram	gram	gram	gram	cm	count	cm	cm			
Grand mean		9.3	12.3	6,028	48.3	183.9	19.1	232.2	38.2	5.8	42.2	19.2			
Nematode	<i>R. similis</i>	18.7 a	24.4 a	11,812 a	40.7 b	168.9 b	18.0 b	209.4 b	36.8 b	5.7 b	41.1 b	18.8 b			
	Non	0.2 b	0.6 b	435 b	55.6 a	198.2 a	20.2 a	253.8 a	39.6 a	6.0 a	43.2 a	19.5 a			
Fungus	<i>F. oxysporum</i>	9.4	12.9	6,934	45.4 b	181.1	19.1	226.5	38.3	5.7 b	42.1	19.0			
	Non	9.1	11.6	5,132	51.2 a	186.8	19.1	238.0	38.1	5.9 a	42.3	19.3			
Soil	Sterile	11.9 a	12.5	5,132 a	43.5 b	174.6 b	19.3	218.0 b	36.1 b	5.5 b	40.1 b	17.9 b			
	Non-sterile	6.6 b	12.0	4,097 b	53.2 a	193.6 a	19.0	246.9 a	40.4 a	6.2 a	44.3 a	20.4 a			
<i>R. similis</i>	<i>F. oxysporum</i>	18.5	24.8	13,228	39.6	168.6	18.0	208.2	37.1	5.6	40.7	18.6			
	Non	19.0	24.0	10,329	41.8	169.2	17.9	210.7	36.4	5.7	41.5	18.9			
No <i>R. similis</i>	<i>F. oxysporum</i>	0.4	0.9	497	51.3	193.6	20.2	244.8	39.5	5.8	43.3	19.5			
	Non	0	0.4	377	59.9	202.7	20.2	262.6	39.7	6.1	43.1	19.6			
<i>R. similis</i>	Sterile	23.7 a	24.7	15,734 a	34.8 c	156.6	18.1	191.4	34.2	5.3	38.8	17.5			
	Non-sterile	13.4 b	24.1	7,612 b	46.9 b	182.4	17.8	229.1	39.5	6.1	43.6	20.1			
No <i>R. similis</i>	Sterile	0.1 c	0.3	11 c	52.1 ab	192.5	20.4	244.7	38.0	5.7	41.4	18.3			
	Non-sterile	0.3 c	1.0	850 c	59.1 a	203.8	20.0	262.9	41.2	6.2	45.0	20.7			
<i>F. oxysporum</i>	Sterile	12.6	13.7	9,007	39.9	167.3	19.0	207.2	35.9	5.3 d	39.8	17.8			
	Non-sterile	6.2	12.2	4,861	51.1	195.1	19.2	246.2	40.7	6.1 a	44.4	20.3			
No <i>F. oxysporum</i>	Sterile	11.2	11.3	6,861	47.0	181.8	19.5	228.9	36.3	5.7 c	40.4	18.1			
	Non-sterile	7.0	11.9	3,324	55.5	192.1	18.8	247.6	40.1	6.2 a	44.3	20.5			

Asterisks indicate significant effect (** $p \leq 0.001$, * $p \leq 0.01$, $p \leq 0.05$); Means for significant effects are indicated in bold font; those with the same letter are not significantly different ($p > 0.05$).

that favor the acquisition of scarce N from soil organic matter (SOM; Gebremikael et al., 2016). Rhizodeposition is part of physiological and morphological changes that include elongation of roots which enable N-starved plants to survive the adverse conditions (Bowsher et al., 2018).

Root infection by *R. similis* appeared to be beneficial to *Fusarium* spp. through creation of wounds and necrotic tissues for saprophytic growth of the fungus (Back et al., 2002; Khan and Sharma, 2020). This manifested in the form of enhanced root piece colonization under the three nutrient deficient regimes (Ochieno, 2010, 2020). *F. oxysporum* V5w2 was expected to cause wilt symptoms (Ochieno, 2020), which are related to pathogenicity of *F. oxysporum* races in susceptible banana cultivars (Dita et al., 2018; Maryani et al., 2019; Segura-Mena et al., 2021). However, there was no basis for linking *F. oxysporum* V5w2 with wilt-related pathogenicity in the N-starved banana plants. This is because the N-starved banana plants were already expressing nutrient stress symptoms including yellowing of leaves and stunted growth (Ochieno, 2010), which also occur in plants infected with pathogenic *F. oxysporum* races (Warman and Aitken, 2018). Possible pathogenic invasion of the N-deficient xylem may not have been nutritively beneficial to endophytic *F. oxysporum* (MacHardy and Beckman, 1981; Pegg et al., 2019), as necrotrophic and saprophytic colonization may have been a more viable nutritional strategy (Orr and Nelson, 2018; Pegg et al., 2019; Ochieno, 2020).

Inoculation of banana plants with *F. oxysporum* V5w2 is expected to suppress *R. similis* multiplication and the associated root damage in banana plants (Kumar and Dara, 2021; Kisaakye et al., 2022). This was the case in the study that utilized N-starved banana plants. Inoculation with *F. oxysporum* V5w2 under conditions of N-starvation was associated with reduced densities of *R. similis* and root damage by the nematode especially in the microbe-rich non-sterile soil. However, endophytic and rhizosphere colonization by *F. oxysporum* V5w2 may have intensified competition for the already limited N and hence inhibiting the multiplication of *R. similis* (Ochieno, 2020). Reduction in optical density of root extracts of banana plants infected with *F. oxysporum* V5w2 and *R. similis* may indicate competition for nutrient resources (Ochieno, 2010, 2020). Therefore, competition for N under its extreme deficiency (Ochieno, 2020), is an alternative to explanations that are based on the concept of induced systemic resistance (Vu et al., 2006; Paparu et al., 2007, 2010, 2013; Sikora et al., 2008; Bubici et al., 2019; Damodaran et al., 2020).

In all the three nutrient starvation regimes, banana plants grown in non-sterile soil had reduced densities of *R. similis* in roots, while root damage caused by the nematode was lower than in sterile soil. The synergistic effect of *R. similis* on root colonization by *F. oxysporum* V5w2 was suppressed in non-sterile soil in the presence of P within the N- and K-deficient nutrient solutions. These were indications of the presence of pest-suppressive microbes in non-sterile soil (Shen et al., 2015; Giné et al., 2016; Jayaraman et al., 2021). For instance, among the microbial genera isolated from banana roots in the present study, some that belong to *Fusarium*, *Trichoderma*, and *Sclerotium* have been associated with nematostatic activities

(Akinsanmi and Adekunle, 2003; Poveda et al., 2020; Kumar and Dara, 2021). Furthermore, *R. similis* may have encountered antagonistic interactions with other nematode genera identified, especially through competition with plant-parasitic nematodes such as *Helicotylenchus multicintus*, *Meloidogyne* spp., and *Pratylenchus goodeyi* (Freckman and Caswell, 1985; Ochieno, 2010). Inclusion of *F. oxysporum* V5w2 in non-sterile soil even caused further reduction in root damage and *R. similis* densities among N-starved banana plants. Elimination of such antagonistic microbes through soil sterilization allowed *R. similis* to multiply and cause root damage. However, suppression of *R. similis* multiplication and the associated root damage in non-sterile soil did not improve the growth of N-starved banana plants. Instead, the N-starved banana plants grown in non-sterile soil were even smaller in terms of biomass and size. Increased demand and hence competition for N by soil microbes including *F. oxysporum* V5w2 in non-sterile soil (Jones et al., 2018), may have limited the growth of the N-starved banana plants and pest-related activities of *R. similis*. Such competition for N was manifested in the form of reduced chlorophyll content in leaves of banana plants grown in non-sterile soil. Inclusion of N in non-sterile soil reversed such trends toward suppression of root damaging activities of *R. similis* while improving banana plant growth, as was evident in the root biomass of plants that received P- and K-deficient solutions that contained adequate N. Therefore, microbial services cannot be well attained under conditions of poor soil fertility and plant nutrition, especially when N is limited. Beneficial soil microbes require proper nutrition to prevent them turning into competitors.

Plants inoculated with *F. oxysporum* V5w2 were smaller in size in terms of leaf development under K-starvation, with most plant growth related parameters inclining toward such trend under P-starvation. This is a pointer to the fact that the fungal pathogen *F. oxysporum* V5w2 may have initiated its plant growth suppressing effects within the short period (Ochieno, 2010, 2020). Under P-starvation, co-infection of banana roots with *R. similis* and *F. oxysporum* V5w2 resulted in increased density of the nematode and higher root colonization by *Fusarium* spp. Such trends in *R. similis* and *Fusarium* spp. densities appeared to manifest in roots of banana plants grown under K starvation. Densities of *R. similis*, *Meloidogyne* sp., and *Pratylenchus* sp. have also been found to increase in roots of *F. oxysporum*-inoculated field-grown banana plants (Ochieno, 2010, 2020, 2021). Such trends in *R. similis* and *Fusarium* spp. densities are indications of nematode-fungi disease complexes (Back et al., 2002; Khan and Sharma, 2020; Ochieno, 2020), which may be favored in soils that are deficient in P and K among other unfavorable ecological conditions (Almeida et al., 2018; Olivares et al., 2021).

Various speculations can be put forward to explain the positive outcomes of interactions between *R. similis* and *F. oxysporum* V5w2 under the P-limited conditions. First, it is worth noting that P is an important nutrient in root development (Crombez et al., 2019; Siedliska et al., 2021). Phosphorus is part of the phospholipids that form the plant cell membranes (Wang et al., 2017, 2021). P-deficiency is associated with weakened cell membranes (Gniazdowska et al., 1999; Pfaff et al., 2020), which may easily be penetrated by *R. similis* and *F. oxysporum*.

V5w2 resulting in increased colonization by the two pathogens. Phosphorus is a chemical component of adenosine triphosphate (ATP; Wang et al., 2021), which provides energy for biosynthesis of plant defensive compounds (Dhakshinamoorthy et al., 2014; Crombez et al., 2019; Desmedt et al., 2020). P-deficiency may limit the biosynthesis of such defensive compounds and render banana plants less resistant to *R. similis* and *F. oxysporum* V5w2. Under P-deficiency, *F. oxysporum* V5w2 may have released phosphatases to enhance acquisition of the limited nutrient from organic sources (Tarañdar et al., 2001; Nunez-Rodriguez et al., 2020). Such enzymes may have further degraded the weakened and damaged roots to facilitate penetration and colonization by *R. similis* and *F. oxysporum* V5w2. P-deficiency may also have triggered increased root exudation of organic carbon in the form of sugars into the rhizosphere (Louw-Gaume et al., 2010, 2017; Canarini et al., 2019). The organic carbon may have intensified rhizosphere colonization by the saprophytic *F. oxysporum* V5w2 (Ochieno, 2020; Clocchiatti et al., 2021), making root invasion, colonization and degradation even more severe for easier penetration and multiplication of *R. similis*. Potassium on the other hand has been considered to have multiple roles including plant defense against pests and diseases (Amtmann et al., 2008; He et al., 2020). K-starvation may have interfered with mechanisms related to plant disease resistance to *F. oxysporum* V5w2 resulting in poor growth.

Soil sterilization exerted various effects on nematode-fungus-banana root interactions that are not only related to the removal of beneficial and pathogenic microbes (Li et al., 2019), but also to other physico-chemical changes that may have been instigated by the high sterilization temperatures (De Deyn et al., 2004; Lees et al., 2018). In the presence of N, banana plants grown in non-sterile soil had low densities of *R. similis* that caused less death of roots, resulting in better growth in terms of biomass and size. Poor plant growth in sterilized soil may indicate the elimination of beneficial microbes in the endospheres and rhizospheres (Ochieno, 2010; Kaushal et al., 2020), including nematode-suppressive microbes, arbuscular mycorrhizal fungi (AMF), phosphate solubilizing microbes among other beneficial soil biota (Paliwoda and Mikiciuk, 2020; Van der Veken et al., 2021). However, the poor growth of banana plants in the steam-sterilized soil may also have alternative explanations. For instance, it is possible that soil sterilization led to decreased bioavailability of P through heat-driven sorption (Shaw et al., 1999; Sinegani and Sedri, 2011; Bai et al., 2017), or even made the fixed P more water-soluble and hence prone to loss through leaching (Shaw et al., 1999; Sinegani and Sedri, 2011; Bai et al., 2017), with the condition being made more extreme through supply of the P-deficient solution. Despite the explained presence of beneficial microbes, root necrosis was higher in P-starved banana plants grown in non-sterile soil. This raises questions as to why root necrosis did not relate directly with *R. similis* densities and root death that instead appeared to have been suppressed. Evidence from the data presented in the present study shows that *R. similis* and soil sterility exerted independent activities toward root necrosis under the P-deficient conditions. It is likely that other root

parasitic nematodes and root-rot microbes in non-sterile soil contributed to the development of the necrotic lesions under the P-limited conditions (Ochieno, 2010; Finkel et al., 2019). This may have involved the secretion of microbial phosphatases for the acquisition of P from disintegrating root tissues (Tarañdar et al., 2001; Cabugao et al., 2017, 2021).

Despite all these speculations having considerably valid bases, consensus toward the outcomes of interactions between *F. oxysporum* V5w2 and other fungal phytopathogens with plant-parasitic nematodes in the banana rhizosphere context (Ochieno, 2020), are complex matters that still exist within a conundrum of diverse views in plant sciences (Schulz and Boyle, 2006; Ochieno, 2010, 2020; Avedi et al., 2014; Taylor, 2014; Schouten, 2016; Brader et al., 2017; Maryani et al., 2019; Geiser et al., 2020; Ochieno et al., 2021). This requires more investment toward research on the diversity and function of root and rhizosphere microbiomes under different ecological conditions (Li et al., 2019; Berg et al., 2020; Kaushal et al., 2020). Proper identification of *F. oxysporum* V5w2 among other strains that have been proposed for biological control requires whole genome sequence (WGS) analysis to differentiate them from pathogenic ones within the Fusarium of Banana Complex (FOBC; Kurtz, 2010; Schouten, 2016; Maryani et al., 2019; Bedoya et al., 2021). Characterization of interacting endosphere and rhizosphere microbes and invertebrates could be strengthened with better methods such as those based on metagenomic DNA sequence analyses (Sharpton, 2014; Nesme et al., 2016; Zakeel and Safeena, 2020). Metabolomic analyses of root exudates based on gas chromatography-mass spectrometry (GC-MS), liquid chromatography (LC) platforms or nuclear magnetic resonance (H-NMR) would have provided further details on changes instigated due to banana root interactions with rhizosphere biota and nutrients among other factors (van Dam and Bouwmeester, 2016; Vives-Peris et al., 2020). Changes in rhizosphere microbe populations in the form of colony forming units (CFU) could be monitored through plate dilution method of plant and soil samples (Vieira and Nahas, 2005), or by other multiple culture-independent measurements including adenosine tri-phosphate (ATP), flow cytometry (FCM), quantitative real-time PCR (qPCR), phospholipid fatty acids (PLFA), and microbial biomass carbon (MBC) (Li et al., 2008; Zhang et al., 2017). Follow-up studies may as well explore the possible mechanisms of *R. similis* suppression in the non-sterile rhizosphere, which may include direct parasitism or predation on the nematode or its eggs (Sikora et al., 2008; Li et al., 2015), antibiosis through production of toxic metabolites (Mwaura et al., 2010; Keswani et al., 2020), competition for nutrients and other resources (Kerry, 2000), as well as induced systemic resistance (Sikora et al., 2008; Walters, 2009; Paparu et al., 2013; Poveda et al., 2020). Such studies may be complemented with those assessing microorganisms that assist the survival and parasitism of plant-parasitic nematodes by protecting them against antagonistic microbes (Topalović and Vestergård, 2021). It should also be considered that microbes such as arbuscular mycorrhizal (AM) fungi known to be beneficial to host plants may also suppress plant growth while increasing infections by plant-parasitic nematodes (Frew et al., 2018). In all these, it remains

an open question on whether development and evaluation of resistant banana cultivars should focus on independent pest species (Tripathi et al., 2015; Seenivasan, 2017; Rebouças et al., 2018), or whether a broader approach that tries to simultaneously tackle disease complexes and abiotic stresses would be achievable in providing better banana cultivars (Lorenzen et al., 2010; Almeida et al., 2018; Batte et al., 2019; Tripathi et al., 2019, 2020; Rocha et al., 2020).

In summary, results from the present three experiments have shown that soil microbes provide ecosystem services through pest and disease suppression while promoting banana plant growth. Soil sterilization and tissue culture eliminates such beneficial microbes to enhance root infection and damage by *R. similis* and *F. oxysporum* V5w2. Plant growth promoting benefits of the soil microbes are negated by nutrient stresses, especially N deficiency that shifts their activities toward being competitors that suppress banana plant growth. However, under such extreme N-deficient conditions, pathogenic *F. oxysporum* V5w2 and *R. similis* may create some yet to be clearly understood impression of banana plant growth promoting services. Competition for N under extreme starvation of this very important nutrient explains the suppression of *R. similis* by *F. oxysporum* V5w2, but not the widely claimed induced systemic resistance mechanisms in banana plants. On the other hand, P deficiency appears to provide physiological conditions that favor synergistic interactions between *F. oxysporum* V5w2 and *R. similis*, whereby the densities of both pathogens in banana root tissues are increased. Potassium deficiency appears to provide physiological conditions that favor pathogenicity of *F. oxysporum* V5w2 by constraining plant growth, especially in terms of leaf development. These are part of issues that could inform the application of microbial services in Sustainable Food Systems (Timmis et al., 2017), particularly in the pending decision on registration of *F. oxysporum* V5w2 as a commercial biopesticide (Dubois et al., 2006, 2011; zum Felde, 2011; Ochieno, 2020, 2021; Kisaakye et al., 2022), in line with International Standards for Phytosanitary Measures (ISPM) among other related policies (Wabule et al., 2003; Avedi et al., 2014; KEPHIS, 2014; FAO-ISPM, 2021).

In conclusion, pathogenic interactions between *R. similis* and *F. oxysporum* V5w2 are predominantly suppressed by a complex of soil microbes that exert plant growth promoting effects in tissue culture banana plants through N, P, and K dependent processes. Nitrogen is the most important limiting factor in rhizosphere interactions between banana roots, beneficial microbes and the pathogens. Soil sterilization and the stringent aseptic tissue culture techniques still require the development of alternative innovative ways of conserving microbial services for sustainable agriculture.

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DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because the conflict of interest as declared only makes it possible to refer to data that was jointly published in Ochieno (2010). Requests to access the datasets should be directed to dwochieno@gmail.com.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Conflict of Interest: This work has been disassociated from studies that propose *Fusarium oxysporum* V5w2 as an Endophytic Biological Control Agent and the matter has already been openly declared (Ochieno, 2020). Prof. Marcel Dicke, Prof. Arnold van Huis, Dr. Thomas Dubois, Dr. Danny Coyne and Dr. Piet J.A. van Asten have renounced their co-authorship due to difference of opinion about the interpretation of the data and the conclusions of this study. My current employer

who is Masinde Muliro University of Science and Technology (MMUST) with its Centre for African Medicinal Flora and Fauna (CAMNFF) was not involved in the research described in this article.

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